Studies on Ceylon Hæmatozoa.

No. 1.—The Life Cycle of Trypanosoma vittatæ.

By
Muriel Robertson, M.A.,
Carnegie Fellow in the University of Glasgow.

With Plates 16 and 17 and 4 Text-figures.

CONTENTS.

I. Introduction.

In August, 1907, I went to Ceylon with a view to investigating the Protozoan blood parasites of Reptiles. The following memoir gives an account of the Trypanosome infection found in the soft Tortoise Emyda vittata.

I wish here to express my great indebtedness to Dr. Arthur Willey, F.R.S., Director of the Government Museum in Colombo. He not only obtained laboratory facilities for me at the museum but also gave me the most enthusiastic assistance in every possible direction. In some instances, at very considerable inconvenience to himself, he actually sent me the infected material which he had come across while engaged upon other work in the jungle. Indeed it is to him, as will appear later, that I owe the discovery of the true intermediate host in the infection which forms the subject of this paper.

Emyda vittata is a soft tortoise covered all over with soft skin, coloured black above and pure white underneath, hence its native name of "kirri ibba" or milk turtle.

The animal has flaps on the under side so arranged that all the four limbs and the head can be completely withdrawn. Like most of this group it is more or less nocturnal in its habit, it is said to leave the water very rarely, but I have myself, while watching a pool near Allutoya, where the country is all jungle, seen one come out of the water immediately after sunset and start prowling about at the edge. These soft tortoises die if they are kept out of water for more than a few hours, so that in order to carry them safely from one place to another it is necessary to wrap them in a damp cloth or put them in a wet bag of sacking or palm leaf. The creature is to be found practically all through the low country. I examined specimens from Colombo, from Kesbewa and Hanwella (both not far from Colombo) from Hambentot, which is on the eastern side, and from Trincomalee, also on the eastern side. It is, relatively speaking, common, but not nearly so abundant as the lake tortoise, Nicoria trijuga, as far as I could make out they were on the whole more plentiful on the eastern side than in the west.

I never heard of Emyda vittata being seen up country, although Nicoria trijuga is to be found in large numbers at Peradeniya and in the Kandyan district generally, which is at an elevation of nearly 1500 feet.

Emyda vittata is very generally infected both with a large Trypanosome (Plate 16, figs. 1—4), and with a Hæmogregarine. I never came across a really satisfactory negative case, although cases were observed where one or other of the parasites appeared to be absent. Protozoologists are quite familiar with the difficulty of being certain that a negative diagnosis is correct. I several times tested apparently negative specimens only to find that at last a stray parasite or two did finally turn up.

I attempted to obtain uninfected specimens by hatching out the eggs already provided with hard shells which were

found in the oviduct of a freshly-captured Emyda. There were five eggs but none of them hatched out, and Mr. Fernando, the taxidermist of the museum, who had, he told me, frequently tried similar experiments, said that he had never got eggs obtained in this way to hatch out. It might possibly be that the length of time that the egg spent in the oviduct before being laid had some effect upon its capability for further development. One of the eggs was opened after more than three weeks, and although it was quite well preserved no development had taken place.

As a rule the Emyda was fairly well infected and in some cases the blood contained a very large number of parasites. I may say at the outset that in spite of the most diligent search and of a slight theoretical bias in favour of the hypothesis I could not find any connection between the Trypanosome and the Hæmogregarine infections. I propose to discuss the Hæmogregarine in a subsequent paper.

As far as my observation goes this Trypanosome is only found in Emyda vittata and I have called it Trypanosoma vittatæ. A Trypanosome is found in the fish Saccobranchus (see "Some Ceylon Hæmatozoa," Drs. Castellani and Willey, 'Quart. Journ. Micr. Sci.,' vol. 49, 1905), which inhabits similar localities, but this is obviously a quite distinct species. Nicoria trijuga, though often to be found living side by side with Emyda vittata, and also as in the Colombo lake in water which harboured a very large number of the generally infected Saccobranchus, never showed any sign of a trypanosome infection at all. My own observations upon blood parasites in Ceylon and those of many observers upon the European forms, especially those occurring in birds and amphibians, point towards the necessity of exercising considerable care before deciding that any hæmatozoon is specific to any given vertebrate host. Nevertheless, in the present instance I feel considerable confidence in attributing the Trypanosome in question exclusively to Emyda vittata.

Trypanosoma vittatæ is a large form resembling

T. raiæ in its external appearance and partly also in its movements in rather a remarkable manner, the most striking difference being in the situation of the trophonucleus which in T. vittatæ lies much nearer to the kinetonucleus.[1]

II. Observations upon the live Trypanosome.

A great deal of time was spent in making observations upon the living object, as it is obvious that where possible it is by far the most satisfactory method.

Trypanosoma vittatæ in the live state is a pyriform organism with a well developed frilled membrane. The frilled appearance is of course due to the membrane being longer from tip to tip at the free edge than at its origin from the protoplasmic body. The trophonucleus can be clearly distinguished as a circular body lying at no great distance in front of the kinetonucleus. It appears as a greyish sphere surrounded by a brighter halo: there is something very characteristic in the rather soft way in which the nuclear structures refract the light, contrasting sharply with the very hard, bright appearance of the protoplasmic inclusions—this is alike true of Trypanosomes and Hæmogregarines. Striations

[1] I have adopted the now very generally accepted terms of kinetonucleus and trophonucleus for the small and large nuclear bodies respectively. These terms seem to me to express more adequately than any of those hitherto proposed the nature and function of these two structures. In this paper the expression "anterior" end is used as equivalent to the flagellate end, "posterior" end as equivalent to non-flagellate end. The evidence in favour of this view being correct seems quite convincing when one has regard to those Trypanosomes in which the Trypanosome phase is derived from a Crithidial or Herpetomonad form in the normal life cycle. The evidence for regarding the flagellate end as the anterior is not so clearly indicated in Trypanosomes which adopt the crithidial or herpetomonad condition by the mere alteration in shape of the body and the migration towards the flagellate end of the already existing flagellum. This development, as is well known, is said to occur in the cultured forms of a very large number of different Trypanosomes, notably those of birds and mammals.

or myonemeta can be seen running longitudinally along the body; these are sometimes extraordinarily clear, and are especially conspicuous just before the animal rounds itself off.

The flagellum runs forward from the kinetonucleus—which can occasionally be distinguished in the live specimen—along the edge of the undulating membrane and ends in a long free whip. The waves of contraction which pass along it make little sharp corners appear at what may be called the bays of the frills, giving a very characteristic appearance, though one difficult to describe in words.

Bright inclusions may be present all along the body, arranged without any appearance of regularity; they are often entirely absent, and I never discovered upon what either their absence or their presence depended. The body of the Trypanosome is apparently oval in section. The posterior end extends some way beyond the kinetonucleus; it is changeable in shape, and may be drawn out to a rapidly tapering point, or be rounded off and rather blunt.

The movements of this Trypanosome are rather complicated, and it can execute a considerable number of different figures. Like many Trypanosomes its motion of translation in the blood of the vertebrate host is relatively speaking slight; this is in marked contrast to the extraordinarily rapid darting movements of certain of the forms developed in the transmitting host. Quite possibly this lesser power of actual translation through space is correlated with the fact that the parasite in the vertebrate blood is in a medium which is itself in motion. Trypanosoma vittatæ shows the wheel motion so often seen in Trypanosomes in fishes; it also executes repeated serpentine twisting, sometimes in a figure 8, or even following a simple U-curve down one limb and up the other. The most characteristic movement, however, is a rather slow, forward spiral twisting. The Trypanosome will sometimes go on revolving slowly round its long axis with the body in the shape of a corkscrew, and with hardly any forward motion at all. The spiral twisting often occurs rhythmically forwards and backwards, through a distance of about

twice or thrice the length of the Trypanosome. This last movement is familiar to observers who have worked with Spirochætes, only in these it is intensely rapid, while in the case of the Trypanosomes it is quite a slow movement.

In most infections small Trypanosomes (Pl. 16, figs. 5—7) are to be seen, less than half the size of the average specimens. In these the membrane is generally a little wider relatively than in the case of the larger forms, and the part of the body posterior to the kinetonucleus is much shorter and generally more pointed. The protoplasm is usually rather hyaline. I do not think that these small specimens belong to a separate species, as forms intermediate in size are also to be seen. Further, these small creatures take part equally in the developmental process to be described presently. I am, however, ignorant of their origin.

Another variation, only rarely met with, is that some specimens have the trophonucleus very much further forward than is usual. These creatures were present in small numbers only in one infection. The ordinary forms were also present. I cannot be certain as to whether they belong to the same species or not, but am personally inclined to think that they do.

There is always a considerable variation in the size and thickness of the Trypanosomes, and also, to a certain extent, in their staining reactions; but it is not marked enough for there to be any reason, in my opinion, for dividing them into male, female, and indifferent upon their morphological characters. This type of difference between the forms is much less evident than, for instance, in such a Trypanosome as T. brucei.

Longitudinal division does occur, but it is only very rarely to be seen, even in good infections. Specimens with two trophonuclei are very occasionally to be seen; it so happens that I have seen these chiefly among the intermediate sized forms.

If blood infected with Trypanosoma vittatæ is placed upon a slide, covered with a coverslip and sealed with vase-

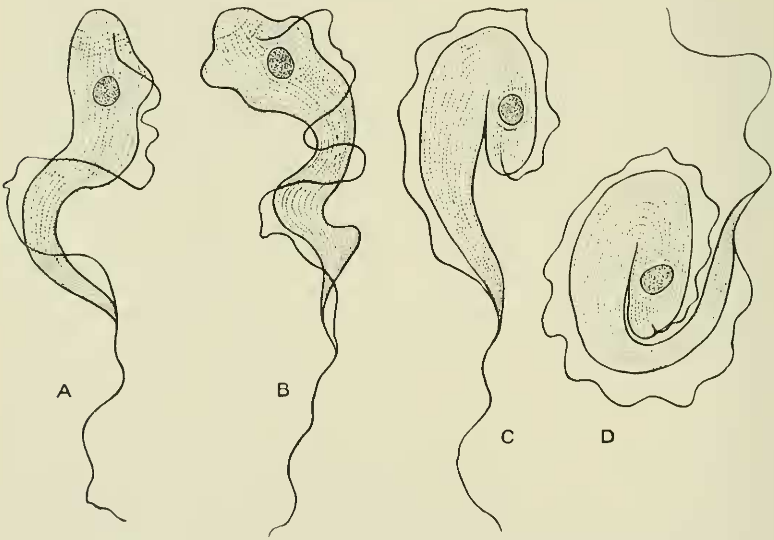
line, quite a different form of multiplication can be observed. The first time I observed this, a slide with blood very strongly infected with Hæmogregarines, and only relatively slightly with Trypanosomes, had been left overnight. Various alterations in the appearance of the Trypanosomes, to be described presently, had been noted before leaving the slide in the evening. Early next morning the slide was found to contain quite a number of small flagellates, just about the size of the Hæmogregarines and very much smaller than the Trypanosomes. A few unaltered Trypanosomes were present, but no intermediate forms. The appearance of the flagellates strongly suggested a connection with the Hæmogregarines. They had comparatively short flagella, the membrane reaching to about between one third and two thirds of the way up the protoplasmic body in different specimens. In fact, they showed a tantalising resemblance to the figure of the Trypanosome phase in the blood of the little owl, as described in Schaudinn's well known memoir, and attributed by him to the life-history of Proteosoma noctuæ.

The experiment was repeated several times, and the following development was made out, showing clearly that the organism arose from the Trypanosome.

Some time after making the preparation the Trypanosomes begin to show various modifications in the external appearance. The length of time which elapses before the creature begins to yield to the altered conditions is remarkably variable, the time co-efficient throughout the whole process is in fact very inconstant. Generally speaking the organisms remain unaltered for about an hour and a half. The alterations in appearance culminate by the complete loss of the Trypanosome shape and the rounding off of the organism, but this condition is arrived at in various ways. Some of the Trypanosomes simply become much thickened at the non-flagellate end. Many become bent upon themselves, and the two limbs of the bend then fuse together (text-fig. 1, C and D). The text-figures illustrate these appearances. The myonemata become much more evident in most cases during

these early phases. In some cases the animal broadens considerably and adopts the spiral shape, the turns of the spiral fuse together, and the most grotesque dumpy creature is produced, which keeps up a slow corkscrew or revolving motion (text-fig. 2).

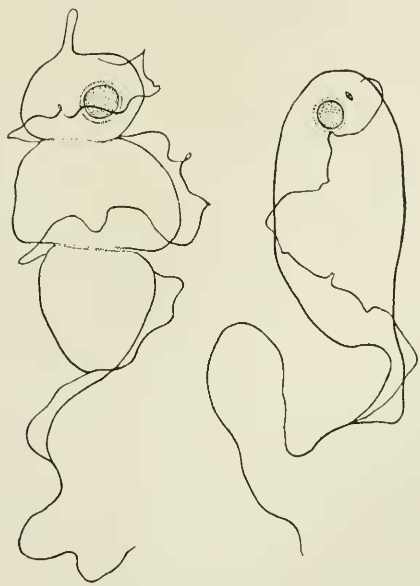
Another appearance of rather a curious character is that where the screw movement backwards and forwards is kept up but very slowly, and the body no longer preserves its



TEXT-FIG. 1.—Sketches of live Trypanosomes to show early phases in the rounding off of the parasites. A and B are one individual, so also C and D.

regular fusiform shape, but bulges now in one direction now in another (text-fig. 1, A and B). The movement is difficult to convey, but is best described as very metabolic euglenoid movement associated with a slow screwing backwards and forwards. During this movement the myonemeta can still be very clearly seen, and besides these at the non-flagellate end circumferential lines running round the creature can be seen, especially during the screw forward.

These appearances seem on the surface to show a curious amount of variation, but it is easily explained if it is remembered that at this stage an obvious decrease in the firmness of the peripheral protoplasm is taking place; in fact it becomes much more soft and viscid. This in correlation with the various methods of movement found in the normal trypanosome produces all the figures noted above. Thus, for

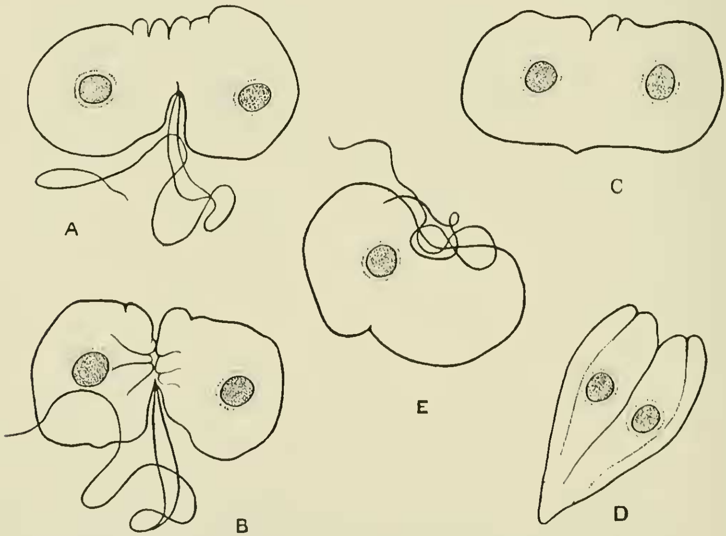


TEXT-FIG. 2.—Two different methods of rounding off.

instance, in text-fig. 1, C and D the Trypanosome has obviously been executing the wheel motion when its protoplasm began to soften, and fusion occurred, and so on.

Finally, whatever the method adopted the Trypanosome comes to rest, and the flagellum breaks loose from the membrane while retaining its attachment at the kinetonucleus. It still lashes about for a time. All trace of the myonemeta completely disappear, and the animal appears as an irregular mass of protoplasm (text-fig. 3, E). Occasionally

part of the body at the extreme anterior end (flagellate end) projects—this is, as far as I could make out, not withdrawn into the body, but seems like the membrane and flagellum to disintegrate. After a time the nucleus becomes quite indistinct, and I noticed that after this I was never able to get a very clear view of the nucleus until just before the flagellate condition was again adopted, when it showed with customary distinctness. Furrows now begin to appear in the animal, and it divides into two (text-fig. 3, A and B). Another division



TEXT-FIG. 3.—Stages in the crop of the leech. A and B. Division stage of one individual. C and D. Individual adopting the pear shape at time of division. E. Newly-rounded off Trypanosome with flagellum still attached.

follows this, and four irregular rounded or pear-shaped creatures are thus formed, lying generally more or less connected. They now put out each a flagellum on one end. The flagellum is at first simply a short thick process. It lengthens and begins to lash slowly from side to side, but is as yet not capable of moving the body. Presently a slight oscillation of the body of the parasite is to be observed, and ultimately as the flagellum lengthens the creature becomes motile.

On one occasion I observed the whole process under a high power in an already pear-shaped individual. The flagellum can only be said suddenly to have appeared as a short, relatively thick process at the blunt end of the organism. This lengthened and became motile, and after a time its origin from the body appeared to lie more laterally, and a slight ridge became visible at that point. I am inclined to think that the ridge is the first appearance of the undulating membrane.

At this stage again, both as observed on the slide and when the process takes place, as will be seen later, in the leech, much variation in small detail is to be remarked, especially in relation to the relative times at which the different processes occur. Thus in the present case the preparation for the second division may, and very often does, take place before the completion of the first. Or, on the other hand, the two products of the first division may become quite separate before any preparation for the second division can be detected. In the matter of the flagella there is also much variation. Sometimes all the four flagella are developed before the first division occurs, or this may not take place until the completion of the second division. Generally speaking, the development of the flagellum lags behind when the process occurs on the sealed slide, while in the leech the flagella are developed as a rule very early.

The typical pear shape, which ultimately becomes fusiform, may be adopted very early; in fact, sometimes at the second division the protoplasmic body will adopt the form of a longitudinally-furrowed cone rounded at the broad end. These furrows are rather curious, as there may be a number of them giving the animal a ridged appearance. The deepest furrow is where the ultimate line of division occurs. The other furrows disappear. The length of the flagellum is again in some cases considerable before the body of the organism begins to lengthen at all, and rounded little creatures, with quite long flagella, may not uncommonly be seen in blood from the crop of the leech; they are of quite

rare appearance on the sealed slide of blood direct from the Emyda vittata.

At this stage of the investigation the question of the transmitting host came to be considered. This was the more difficult to determine as no parasites of any kind had been found on the milk tortoises. The aquatic habit of the tortoise pointed to some water inhabiting blood-sucking form, and of these the leeches seemed the most likely. A number of the common Ceylon water leech, called by the natives Diya kudella,[1] were investigated, but none of them showed any sign of the trypanosome. It was found, however, that the leech fed readily upon the tortoise. Upon puncturing the crop immediately after feeding it was seen that the Trypanosomes began to undergo the above-described transformation at once upon being taken into the crop.

Thus a leech was put on to a milk tortoise at 8.30 a.m.; at 10.30 it was still feeding; at 11.30 it was found free in the tank. The crop was punctured at once, and the blood examined in the usual way on a slide ringed with vaseline. The Trypanosomes were for the most part already casting off their flagella, and many of them had already undergone the first division. By 1 o'clock they were divided into four, and many of the resulting Herpetomonas forms had become free, and were swimming about on the slide. Blood was then taken from another part of the crop, and the state of the parasites was exactly similar to that on the sealed slide.

The puncturing of the leech does not apparently cause it much inconvenience. The specimen mentioned lived quite well till the next day, when I finally opened it at 7.30 a.m. Many actively motile forms in the Crithidial stage were to be seen.

Further divisions it seems may occur after the two mentioned, and also a secondary increase in size. Some of the individuals had become considerably lengthened, and were

[1] Mr. W. A. Harding, who kindly examined the leeches brought from Ceylon, considers this leech to be Limnatis (Poecilobdella) granulosa.

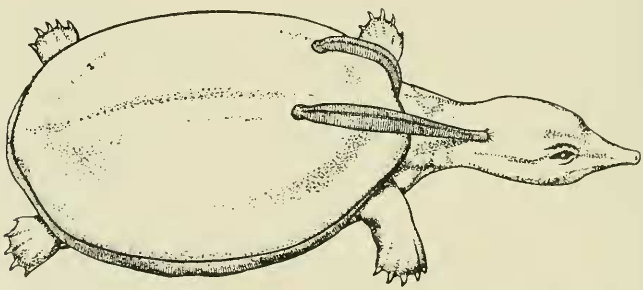
rather slender, but in almost every case the trophonucleus was posterior to the kinetonucleus.

Another leech killed ninety-five hours after feeding on the same tortoise showed slender Crithidial forms in the crop and some irregularly-shaped individuals. The intestine never showed many of the flagellates. A number of leeches were allowed to feed on various tortoises with the intention of observing the progress of the infection at different distances of time. Unfortunately, the work was at this point interrupted by illness for a number of weeks. On resuming it I found that the Trypanosomes still did persist in the crop in a few cases, but they were always very scarce. In one positive case they were to be found six weeks after feeding. An experiment was tried by feeding a leech for a second time on infected blood to see if the second infection would persist in greater numbers than the first, but by some fatality the tortoise ate the specimen that I had in this condition, and I never made out if the parasite showed any signs of becoming acclimatised to the host. The tortoise showed the greatest desire to eat the leeches, and had to be carefully watched; even so, it was surprising how often the more lively Emydas got the leech. They gulp them down, but do not break the skin with their teeth. The leech seems rather difficult to swallow, but the tortoise is most persevering, and it is almost impossible to rescue the leech once the tortoise has got well started.

I mention this, as there is always the possibility of parasites being spread by way of the digestive tract. The leech generally prefers to sit on the carapace attached by the posterior sucker and to fix its anterior sucker to the occiput, or back of the neck, or into the humeral angle. The accompanying text-figure is from a pencil sketch made in a few minutes by Dr. Willey, and gives a very typical picture. The leech will stretch to an almost incredible extent when the tortoise puts out its head rather than let go.

There is one point in the habits of this leech worthy of mention, namely, the fact that it will often feed in two instalments. Thus a leech would attach itself and feed for

about an hour, and then cease and move about on the carapace, or even leave the tortoise altogether, and upon being replaced would take a second meal. I was in some doubt as to whether the leech had always fed the first time, but in a few cases it certainly had, and it had generally made the characteristic little wound. This habit, I think, may have a certain importance, owing to the very rapid changes which take place in the Trypanosome in the crop. I do not wish to imply that the horse-leech is likely to be a facultative transmitting host for this particular Trypanosome in nature, but the fact is of interest in regard to the transmission of parasites by leeches generally.



TEXT-FIG. 4.—Poecilobdella granulosum feeding on Emyda vittata. (Drawn by A. K. Maxwell from a sketch by Dr. A. Willey.)

The time taken to digest a meal appears to be much shorter in the Ceylon freshwater-leech than, for instance, in such a creature as Pontobdella muricata, the common marine leech which infests the skate of European seas, where very many months elapse before digestion is complete. Thus one of the Ceylon leeches which had fed vigorously so that it had been seen to swell out to a great extent, was nearly empty as regards its crop after fifty-three days. The size of the leech has, of course, a good deal to do with the length of time taken to digest a meal, a big leech taking longer than a small one. I should say that about two to six months was the time taken to complete the digestion of a meal. I have no idea how long elapses in nature between the completion

of digestion and the searching for more food. All the leeches captured that I opened were empty of blood, but they could not by any means always be made to feed—I opened some of the ones that had refused, and found that they were empty. On the other hand a leech which had obviously fed on December 2nd fed again on February 1st: this beast was eaten by the tortoise, but it seemed to have been feeding and certainly left a wound. Curiously enough all the land leeches I examined were empty, and that was also Dr. Willey's experience in the jungle. This is possibly due to the fact that the fed leeches are not in search of prey, and therefore not so easily found.

On one occasion a curious example was given of how indifferent the water-leech is as to whether it feeds upon warm- or cold-blooded creatures. I had tried to make one of them feed upon a Saccobranchus infected with Trypanosomes with a view to discovering if any such change occurred as that seen in T. vittatæ, the leech steadily refused to feed. I then took it out of the tank, and it instantly attacked my hand making the customary little triradiate scar and drawing blood. It was made to desist, and then immediately put on to a tortoise where it quite contentedly made a large meal.

The experiments with the Diya kudella were now abandoned as what appears to be the true intermediate host was come upon.

Dr. Willey, while making some observations at Kesbewa, about eight miles from Colombo, found three Emyda with a number of small leeches attached to the back of the neck, the angles between body and limbs, and the region near the tail. He very kindly brought them to me at once. The leech is a very small creature. It attaches itself to the tortoise, but is most capricious about staying on it; I found it a most troublesome little animal to work with on that account. It is not very easy to keep it alive in captivity.

The leech belongs apparently to the genus Glossiphonia, and has a trick of lying together in little clumps; it is able to swim, and also covers the ground rapidly by walking

under the water on its suckers in precisely the same way that the common land leech does on land.

This leech broods its young, but it usually seems to carry about only very few with it. I have frequently got them with one or two quite good-sized young ones, and remember on one occasion taking three parents, each carrying one young one, from a tortoise, and putting them into a watch-glass. They had all got detached and mixed up in the process, a short time later they were once more arranged in pairs, but I had no means of discovering if each parent had selected out its own offspring or had just adopted the first one it met.

The Glossiphonia is a very inconspicuous creature, is quite aquatic, and dies very soon if left out of water, which probably accounts for it not having been found sooner. The tortoises were generally brought in by natives, and it was always some hours before they were examined, and I expect in many cases the leeches had died and dropped off before they reached the museum.

From this time forward leeches of this species were got from time to time, but it was difficult owing to the relatively free habit of the beast and small size to get them in numbers. A good number were got for me by Dr. Willey from Hambentot in the south-eastern part of the Island. They came from milk tortoises (Emyda vittata) living in a bathing place; alongside of them were lake tortoises (Nicoria trijuga) with Ozobranchus[1] upon them.

The leeches seem to be specific to the two tortoises, only one Glossiphonia, an empty specimen, was found on the top of the carapace of a Nicoria; its presence was probably quite casual, just as I have occasionally found a stray Branchellion on a Tropidonotus (water-snake) which was living in a tank with Nicoria—they never fed on the snake.

The digestion in the Glossiphonia completes itself in about two to six days, according to the size of the leech, but I do not know exactly what period of time must elapse before the

[1] Ozobranchus, a species of this genus of leech is found in great numbers upon Nicoria trijuga. Mr. Harding states that this is a new species.

animal feeds again in nature. An apparently empty leech will sometimes quite refuse not only to feed, but to remain on the tortoise. Nevertheless, from observations on captive leeches, it does not appear to me to be more than a few days. The Glossiphonia show a marked tendency to get into the less-exposed corners of the body, such as the folds of skin at the back of the neck, round the limb bases, and under the tail. They were actually seen to enter the cloacal chamber which is a relatively large cavity in these tortoises.

The Glossiphonia, in contrast to the horse-leech, shows the Trypanosome very frequently in nature, in fact, the majority of the specimens are infected. And the parasite persists in empty leeches where no coloured matter is to be detected in the alimentary tract. I was never able to find the very earliest stages of the parasite in this leech owing to the difficulty of manipulation. It was neither easy to get the leech in a condition willing to feed nor to catch it at exactly the right moment after feeding. This was, of course, due to its small size and wandering habits correlated with the exceeding rapidity of the early changes in the Trypanosome.

In the most recently-fed animals at my disposal the parasite was already in the shape of a rather broad flagellate approaching the crithidial condition—the first two divisions had, in most cases, already occurred.

Thus a Glossiphonia which had fed on infected blood at some time between 8 a.m. on April 6th and 7 a.m. on April 7th was opened just after the latter hour. The Trypanosomes were already mostly in the shape of crithidia, but a very few were still in the rounded state just completing division. Some long slender forms, very narrow, with pointed posterior end, and the flagellum only reaching back to a little more than the middle of the body were already present. This indicates that the development is even more rapid than in the horse-leech.

These long forms were not, I think, left over from the previous meal as they were of a type not usually found at the end of digestion.

The course of the infection in the Glossiphonia appears to

be in brief as follows:—The Trypanosome ingested with the blood develops in a few hours into a flagellate, rather rounded and broad in shape. It may grow very considerably in size, and adopt the Trypanosome condition, i. e. with the kinetonucleus posterior to the trophonucleus. Division still proceeds. Great variation in shape and size occurs in this middle period of digestion, and the relative position of the two nuclei varies very much even in the two products of one division. All stages from the round, rather dumpy crithidia to immensely long and very slender forms moving with great rapidity darting across the field in a flash are to be seen in the crop at the same time.

These different forms will be described in greater detail when the stained material comes to be considered. Division stages may also be seen, and these are often unequal.

Towards the end of digestion the type becomes much more uniform, and slender forms with little protoplasm and flagella hardly exceeding the length of the body seem to dominate to the exclusion almost entirely of other forms. These creatures very often have the kinetonucleus just anterior to and almost embedded in the trophonucleus. They seem at this stage, moreover, to have reached the limit of division as dividing figures were never found. It appears probable that death would now ultimately supervene unless injected into the blood of the vertebrate host.

Conjugation was carefully watched for, but no sign of it was found. It was expected to occur probably immediately after the first divisions in the leech or else possibly towards the close of the middle period of digestion.

The process of conjugation might of course occur at another point of the life-cycle, namely, at the time when the Trypanosome is injected from the alimentary tract of the leech into the circulation of the tortoise. If that were the case it would amply explain the elusive character of this process amongst Trypanosomes, as that is the one part in the cycle of a plasma dwelling form which it is almost impossible to investigate properly.

The question of the surviving of the Trypanosome in the leech has to be considered. It is a matter which is not easy to settle quite definitely, but I do not think that it occurs in this case. Leeches from uninfected hosts showed no parasites. I had a tortoise with only Hæmogregarines in its blood, and I never got Trypanosomes in leeches from this individual. As far as my observation goes (both as regards the investigation of the live creature and of sections) the parasite is never found outside the alimentary tract, and, while to be found in the intestine, is much more a crop parasite than, for instance, T. raiæ in Pontobdella.

How exactly the infection is transmitted to the tortoise in the act of sucking I do not know. But this much is pretty clear, the leech seems to suck by rhythmic contractions, that is to say, the suction is periodically inhibited.

I do not know if the Trypanosome, which is certainly slightly rheotropic, is sufficiently so to swim against the inward flow of blood during the suction time, but it might conceivably be able to do so in the intervals during which this is suspended. Further, the skin of the host must be pierced before suction begins, and it may be during this part of the process that the parasites are communicated to the vertebrate.

The habits of the Glossiphonia seem admirably adapted to the requirements of an intermediate host: its trick of wandering from one tortoise to another, its apparent conservatism in the choice of a host, and the relatively rapid digestion are all features favourable to the spreading of such a parasite as a Trypanosome.[1]

III. Observations on Stained Material.

So much then for the observations upon the live material. The material for staining was treated in various ways; films were made and dried in air, then fixed in absolute alcohol—or they were exposed to osmic vapour and then allowed to

[1] See note on p. 693.

dry—or they were plunged while still wet into corrosive sublimate and acetic acid and treated by wet methods throughout. It is a matter of regret to me that I did not fix more material in this last way. The films were stained by various Romanowsky methods, or, in the case of wet films, in Heidenhain's iron hæmatoxylin or Ehrlich's hæmatoxylin.[1] The Trypanosome was also studied in section from the various organs.

The drying method followed by the Romanowsky stain gives excellent results with certain types of object, but may at times give misleading pictures, more especially with more massive creatures. It is therefore advisable, where possible, to control the results with material treated by wet fixation; osmic films are very valuable, but are open to the same objection in the matter of drying. The drying method seems to flatten and spread out certain types of organism; on the other hand, the wet fixation of blood-films equally certainly causes the parasite to shrink. This is very marked in certain Hæmogregarine phases. Fixation is generally a choice of errors, but by the combination of the dry and the wet method a very fairly accurate idea of the nuclear structure of the organism may be obtained.

Trypanosoma vittatæ (Pl. 16, figs. 1—7) in the blood of the vertebrate host shows dense protoplasm, markedly alveolar with longitudinal striations, corresponding to the myonemata so clearly visible in the live state. These are not always equally conspicuous. There is no doubt that the adult Trypanosome in the blood of the vertebrate is possessed of quite a definite outer sheath or periplast—this is clearly visible in crushed specimens. The myonemata appear to form part of this structure.

The protoplasm has a tendency to stain deeply, whatever the method used; granules and protoplasmic inclusions are never, in my experience, visible at this stage in the stained preparations. In the live state there are sometimes bright globules to be seen in the protoplasm; they are not very

[1] I am indebted to Prof. Minchin for advice on the handling of these wet preparations.

commonly present, and this occurrence is quite casual. They may possibly be of a fatty nature, and be dissolved out by the alcohol with which the slide is treated. Whatever their nature, they have no visible equivalent in the stained preparations. The membrane presents the usual structureless appearance, and takes up the stains very faintly and evenly. In the Romanowsky films, and also, though less frequently, in wet hæmatoxylin material, a line is to be seen on the membrane, just immediately inside the flagellum; it suggests a supporting or skeletal structure and takes up the plasma stain.

The flagellum runs at the edge of the membrane, and can be traced back very close to the kinetonucleus, but not as a rule actually into it. A minute granule[1] can occasionally be seen just at the root, but it is not by any means always visible. The kinetonucleus is rod-shaped; in Romanowsky preparations it shows as a more massive structure than in the wet films treated with Heidenhain. It is always surrounded by a slightly clearer area of protoplasm, but there is no sign of a definite vacuole in its neighbourhood as is described for some of the mammalian Trypanosomes.

In a dried film the Trypanosome is practically presented in one plane, and a certain amount of widening out occurs, this causes a disturbance of the internal structures to a greater or less extent. The form in question during its sojourn in the blood of the vertebrate is, relatively speaking, a massive creature, it is oval in cross-section, as can be seen in paraffin sections from the lung. It is therefore not particularly well adapted for the drying method. In the wet films the trophonucleus shows a large chromatic karyosome surrounded by a clear area, which, in turn, is bounded by a sharply-defined ring, which takes on a nearly black colour with iron hæmatoxylin (Pl. 16, figs. 1 and 2). I propose to use the word nuclear membrane for this outer ring. I merely do this as a matter of convenience; it is not at all clear how far it can be

[1] This corresponds apparently with the blepharoblast of Minchin, 'Q. J. M. Sci.,' vol. 52, 1908.

regarded as a true nuclear membrane in the metazoan sense, and I do not wish the word to be taken as implying homology. I have never been able to see on hæmatoxylin preparations any strands passing from the karyosome to the membrane, although it is a very usual feature in this type of nucleus.

It is to be noted that the protoplasm shows no differentiation round the membrane of the nucleus, that is to say, that in the wet method films stained with hæmatoxylin there is no protoplasmic halo.

A slightly different appearance is presented in the dried films here (Pl. 16, figs. 3 and 4), there is a wide protoplasmic halo which always takes the blue stain of the Romanowsky combination much less deeply than the surrounding cell substance.

Approximately in the centre of this is the karyosome, which is composed of a blue staining substance underlying a reticulum of red staining chromatin. In some specimens strands seem to pass out from the karyosome—occasionally a pale (fig. 3) red ring surrounds the karyosome and strands may be seen passing between them. It is to be noted that this ring is well within the protoplasmic halo. My interpretation of the Romanowsky appearance is as follows:—The red ring corresponds to the membrane (in the Heidenhain preparations) which is generally destroyed in the drying.

What I have called the protoplasmic halo in the Romanowsky picture corresponds to the space between the karyosome and the membrane, but it is much widened by the pulling back of the protoplasm due to the general flattening of the whole organism.

As before noted small specimens are present, the origin of these (Pl. 16, figs. 5 and 6) is still obscure; they may either arise from the large forms by division or they may possibly be the young forms derived by infection from the leech. I am inclined to consider the former explanation as the more probable. It is, however, impossible, to form any very reasonable opinion on the point except from the results of experimental inoculation.

These small forms have hyaline protoplasm staining faintly with the blue of the Romanowsky combination. The protoplasmic halo is not visible in these forms, and in the wet preparation the membrane or outer ring is always rather faint.

The kinetonucleus lies very close to the anterior end of the body which tapers very rapidly to a sharp point. The membrane is relatively wide and the free flagellum is long.

Division stages in the adult Trypanosome must be exceedingly rare, as although a very large number of well-infected films have been searched I have never come across any of the full-grown forms in this condition. Among the forms intermediate between the adult and the small specimens, however, individuals with two nuclei and dividing kinetonuclei are to be found; they are never numerous, and I can say nothing of the details of the process (Pl. 16, fig. 7).

As already indicated I can find no morphological grounds for dividing the adult organism as found in the blood of the tortoise into male, female, and indifferent. There is no evidence to suggest that the small specimens like those shown in Pl. 16, figs. 5 and 6, are males and the large females, and outside of this the difference among the specimens is very slight, and involves apparently only protoplasmic features. This, in itself, is, however, no argument against an ultimate sexual differentiation or conjugation in the intermediate host.

The material from the leeches, I am sorry to say, was mostly fixed by the dry method followed by alcohol, a good deal being fixed by the osmic vapour method to act as a control. The Trypanosome is also to be recognised on sections of the whole leech, but it is difficult to get a very clear and brilliant picture. However, the dry method is better adapted to the thin leaf-like shape of the Trypanosome in the intermediate host.

The detail of the very earliest changes in the Trypanosome upon being taken into the leech is difficult to get in the stained preparations as they occur before the leech has ceased feeding. However, they are sufficiently clear from

the live observations. As soon as the Trypanosome has rolled itself up and cast off its flagellum, the division of the nuclear elements begin, and it is at this stage that they are to be found in material from the crop. This early part of the work has mostly been made out from material from the Limnatis (figs. 8—12).

The two first divisions both of the trophonucleus and kinetonucleus follow quickly upon each other, the details of division being not so clear as in T. raiæ. Probably this is due to the slowness of the process in this last-mentioned Trypanosome, which in conjunction with the rich infections found in the intermediate host make the obtaining of a complete series a comparatively simple matter.

In the division of the trophonucleus of T. vittatæ there is a marked resemblance to what occurs in T. raiæ; in fact, the two processes are quite parallel. We have here also a nuclear division where no equatorial plate is formed, and where (figs. 9 and 10) no differentiation of the chromatin into chromosomes occurs. In both cases there is a well-marked spindle apparatus which appears to bring about the division of the chromatin with only a slight disturbance of its arrangement. So also in both instances the substance of the spindle apparatus is absorbed in the protoplasm and not enclosed in the reformed nuclei.

Fig. 9 shows a stage in this division process, and also fig. 10 in which the kinetonucleus has divided into four already, and both trophonuclei are in the act of undergoing the second division.

The kinetonucleus, however, shows a considerable difference from the conditions obtaining in T. raiæ.

In T. raiæ the kinetonucleus became greatly elongated, and then divided transversely, the products of division often remaining connected by a red staining band. In T. vittatæ the kinetonucleus when dividing splits through longitudinally. Sometimes the split starts at one end of the kinetonucleus, but takes a considerable time before being completed, and it may thus present a horseshoe shape. The

kinetonucleus generally precedes the trophonucleus in division, but this is not by any means invariable. I cannot find any sign of the centrosomal function described by França for the Trypanosome in Hyla arborea.[1] He describes a development of the Trypanosome resembling this early process in the leech, but in the case studied by him the kinetonucleus appears to enter into what he considers definite centrosomal relations with the trophonucleus at the time of division. This is quite absent in T. vittatæ.

As already stated the flagellum develops with no very close regard to the exact stage of the nuclear divisions. It grows out rapidly, and in the stained preparations seems to develop by direct outgrowth from the kinetonucleus. The complicated figures met with at a certain stage in the development of T. raiæ in Pontobdella are here entirely absent. The products of the divisions show a certain amount of variation as regards the state of their development at the time of being set free. Pl. 16, fig. 11, is a very typical example of the more rounded forms, fig. 12 is another, and a third is shown in fig. 12 *a*.

Twenty-four hours after feeding the parasites are all flagellate forms varying considerably in shape, but most of them already showing an undulating membrane, and the kinetonucleus has migrated as a general rule pretty far back though it is still in front of the trophonucleus (Pl. 16, figs. 11—13, 16, and 17). In the Glossiphonia this process is still quicker, and from this time on the infection in this leech begins to show the very wide range of forms so characteristic both of T. vittatæ and T. raiæ.

A review of Pls. 16 and 17, figs. 13—26, will give some idea of the various types present. Long slender forms, some with exceedingly elongated bodies and the kinetonuclei showing considerable variation in their relation to each other, typical Trypanosomes often somewhat broad in shape, and small dumpy forms with short flagella, are all present together at

[1] (" Recherches sur les Trypanosomes des Amphibians," 'Arch. de L'Inst. Roy. de Bact. Camera Pestana,' T. i, Fasc. ii, 1907).

this middle stage of digestion. From such a range of forms it would be a simple matter to pick out creatures suggesting the morphological features which are considered characteristic of male and female individuals. Thus Pl. 17, figs. 23 and 25, might be regarded as respectively male and female; so also Pl. 17, figs. 24 and 26; but until the act of conjugation is observed I cannot see that there is evidence enough to make it clear that the quite obvious morphological difference is the expression of a sexual differentiation.

At this middle stage of digestion dividing individuals are of frequent occurrence. In Trypanosoma vittatæ unequal division at this stage is the rule rather than the exception. The process is figured in Pl. 17, figs. 18 and 19, and the different character of the products is obvious.

The new flagellum unquestionably grows out apart, and does not arise by splitting off from the old one. The splitting of the flagellum, as is well known, does occur in certain Trypanosomes; as, for instance, in T. grayi described by Prof. Minchin,[1] and it is of interest to find two such very different methods holding good in the group.

The new flagellum in T. vittatæ will start growing out from the kinetonucleus before it is obviously divided, though it is generally enlarged. A review of Pls. 16 and 17, figs. 16—20, will demonstrate this question pretty clearly.

As time goes on the nature of the infection undergoes a very marked change; the predominating type of Trypanosome is now found to be a slender rather short form with the flagellum extending only to a short distance beyond the end of the body. The undulating membrane is very narrow. The tropho- and kinetonuclei have entered into close relations with each other, and generally the kinetonucleus is just anterior though closely applied to the trophonucleus. Specimens are found with the kinetonucleus posterior, but these are few in number. The gradual predominance of this type to the exclusion of practically every other can be

[1] "Trypanosomes in Tsetse flies and other Diptera," 'Q. J. Micr. Sci.,' vol. 52, 1908.

followed very clearly in different infections. Thus Pl. 17, figs. 31—33, came from a quite empty leech, and only this type with very slight variations was present. Pl. 17, figs. 18—27, come from a leech in the middle period of digestion, and this final type is just beginning to appear in isolated specimens. In another leech a little further advanced it is more numerous than any other, but not exclusively in possession of the field.

I have never seen division in this type which appears at the end of digestion. I am inclined to think, although there is no actual evidence, that these forms die off unless injected into the blood of the vertebrate.[1]

Exactly what happens to the forms which disappear during the course of digestion is not very clear. The broad forms, however, appear to give rise to such forms as Pl. 17, figs. 29—33, simply by division and direct development. The case of the very long slender forms (Pl. 17, figs. 25, 26, and 28) is more difficult, and the material at my disposal does not throw much light on the question.

IV. General Remarks and Conclusions.

The life-history of Trypanosoma vittatæ shows a close resemblance to that of T. raiæ. Some quite recent observations upon this last mentioned Trypanosome made in November, 1908, at the Millport Marine Station with Pontobdella which I had reared from the egg, have amply confirmed Brumpt's brief sketch of the early stages of the life cycle.[2]

Moreover, skate's blood, which contained the Trypanosome kept upon sealed slides has shown that here also the Trypanosome discards its flagellum and undergoes a number of divisions. The process is similar to that of T. vittatæ, but the rounded non-motile stage seems very much more persistent. In the Pontobdella motile flagellate stages do not

[1] It is very difficult to be certain that division never takes place in this form, but I have never seen any sign of it.

[2] 'C. R. Soc. Biol.,' ix, 1906.

begin to appear for four to six days, although the flagellum itself may be present for some time, apparently more than a day, before it becomes motile. The earliest divisions of the rounded Trypansome, instead of following each other immediately as in T. vittatæ, are here at intervals of at least twenty-four hours. The early stages of development of the flagellum are also very slow.

This process by which the Trypanosome rounds itself off and after a number of divisions produces a definite Crithidial flagellate, appears to me to be more widespread among Trypanosomes of a certain type, notably those from cold-blooded hosts, than it has hitherto been considered.

França observed an analogous process some time ago in the Trypanosome of Hyla arborea, also in T. granulosum.[1] Brumpt describes it for this Trypanosome also in the leech Hemiclepsis, and Dutton, Todd, and Tobey[2] have seen it in Trypanosoma loricatum.

Another point of interest in the life cycle of T. vittatæ is the very marked development of a uniform slender type of parasite at the end of digestion. In T. raiæ this also occurs, though here some rounded forms seem often to persist. A somewhat similar development occurs in T. grayi,[3] where the last stage of the infection shows, however, two slender forms, one of which, a Herpetomonas-form, encysts in the proctodæum of the Glossina, while the other does not encyst, and seems probably to be destined to transmit the infection by inoculation into a vertebrate host.

I need not emphasise the absence of evidence of conjugation. This process if it occurs seems particularly difficult to observe in Trypanosomes, and no quite satisfactory account of it has yet been given for this group of flagellates.

It is a point of only slight interest, but it is curious to note to what an extent the infection is capable of persisting in

[1] 'Bull. Soc. Portug. Sc. Nat.,' vol. i, p. 3, Dec., 1907.

[2] 'Ann. Trop. Med. and Parasit.,' vol. i, No. 3, 1907. (I have not seen the original memoir.)

[3] Minchin, loc. cit.

the Limnatis, which is apparently not a true transmitting host.

The work here recorded was done partly at the Government Museum in Colombo, Ceylon, and partly in the Zoological Laboratory in the University of Glasgow. I am much indebted to Prof. J. Graham Kerr for kind suggestions during the course of the work.

GLASGOW; December, 1908.

NOTE.—It may be objected that no absolute proof has been adduced that the form in the Glossiphonia is T. vittatæ. Absolute proof was not possible from the nature of the conditions, but the evidence seems to me to be very strongly in favour of the identity of the parasites. The correspondence of the early stages in Glossiphonia with those in Limnatis where experimental feeding could be carried out, the final stage in the Glossiphonia at the end of digestion being a slender flagellate and not a rounded-off organism as occurs in the flagellate of non-bloodsucking insects, and the absence of the parasite in the leeches from the tortoise where no Trypanosomes could be found all seem to me to point to the stages in Glossiphonia being true developmental stages in the life cycle of T. vittatæ.

I may add that no flagellates were ever found in the land leech or in Ozobranchus or in Limnatis. Ozobranchus, of which a very large number were examined, lives in exactly the same habitat as Glossiphonia but is parasitic on Nicoria trijuga instead of Euyda vittata. Nicoria never showed a Trypanosome.

EXPLANATION OF PLATES 16 AND 17.

All figures were drawn with the Abbé camera under a 2 mm. Zeiss apochromatic immersion objective. A No. 12 compensating ocular and tube length of 250 mm., giving a magnification of approximately 3600 diameters. This has been reduced by the lithographer to approximately 2400 diameters.

Figs. 1–7.—Trypanosoma vittatæ from the blood of Emyda vittata.

Fig. 1.—Trypanosome from blood of tortoise stained with Heidenhain's iron hæmatoxylin after fixation with corrosive and acetic, wet method throughout.

Fig. 2.—As above, showing a characteristic attitude.

Fig. 3.—Dried Giemsa film of Trypanosome showing protoplasmic halo and red ring round karyosome.

Fig. 4.—Trypanosome as above, showing myonemata and line along undulating membrane.

Figs. 5 and 6.—Small specimens from blood of tortoise.

Fig. 7.—Dividing stage from blood of tortoise.

Figs. 8–12A.—Early stages in crop of leech. These are from the water leech, Limnatis granulosa.

Fig. 8.—Division stage; the four new flagella are already developed; kinetonuclei are dividing by longitudinal splitting.

Fig. 9.—Division stage showing nuclear spindle.

Fig. 10.—Second division occurring before the completion of the first as regards the protoplasm. Both kinetonuclei in act of division, only two flagella so far developed.

Fig. 11.—Rounded flagellate—the product of the divisions of the rounded off Trypanosome.

Fig. 12.—Early flagellate stage; note the lengthening of the body.

Fig. 12A.—Another early flagellate stage.

All the remaining figures are from the Glossiphonia, with the exception of 16 and 17.

Figs. 13–15.—Osmic fixed film from leech just about the beginning of the middle stage of digestion.

Fig. 13.—Flagellate stage showing elongated body.

Fig. 14.—Flagellate, with broad posterior end.

Fig. 15.—Very long flagellate kinetonucleus at same level as trophonucleus.

Figs. 16, 17.—Early flagellate stages from horse leech to show secondary increase in size and preparation for division. Note the condition of the flagella showing outgrowth from the kinetonucleus.

Figs. 18–27 from the Glossiphonia at middle stage of digestion.

Fig. 18.—Division stage. Note relative position of the kinetonuclei and the condition of the flagella. The unequal character of the division is obvious.

Fig. 19.—Another division stage. The features are much as in Fig. 18.

Fig. 20.—Early division stage to show condition of kinetonucleus and flagella.

Fig. 21.—Trypaniform individual with broad posterior end and many red-staining granules in the protoplasm.

Fig. 22.—Small broad form.

Figs. 23 and 24.—Short, rather broad, trypaniform individuals.

Figs. 25 and 26.—Long slender forms.

Fig. 27.—Isolated forms of this type are just appearing in this leech which is at the middle stage of digestion. This is very like the final type developed at the close of digestion.

Fig. 28.—Very long slender form.

Figs. 29 and 30.—From a leech whose digestion is still more advanced Note the difference in the type of the organism.

Figs. 31–36.—Flagellates from leech at end of digestion, this type alone is present with very few exceptions.